Mutational analysis of K28 preprotoxin processing in the yeast *Saccharomyces cerevisiae*

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K28 killer strains of *Saccharomyces cerevisiae* are permanently infected with a cytoplasmic persisting dsRNA virus encoding a secreted α/β heterodimeric protein toxin that kills sensitive cells by cell-cycle arrest and inhibition of DNA synthesis. *In vivo* processing of the 345 aa toxin precursor (preprotoxin; pptox) involves multiple internal and carboxy-terminal cleavage events by the prohormone convertases Kex2p and Kex1p. By site-directed mutagenesis of the preprotoxin gene and phenotypic analysis of its *in vivo* effects it is now demonstrated that secretion of a biological active virus toxin requires signal peptidase cleavage after Gly³⁶ and Kex2p-mediated processing at the α subunit N terminus (after Glu-Arg⁴⁹), the α subunit C terminus (after Ser-Arg¹⁴⁹) and at the β subunit N terminus (after Lys-Arg²⁴⁵). The mature C terminus of the β subunit is trimmed by Kex1p, which removes the terminal Arg³⁴⁵ residue, thus uncovering the toxin's endoplasmic reticulum targeting signal (HDEL) which – in a sensitive target cell – is essential for retrograde toxin transport. Interestingly, both toxin subunits are covalently linked by a single disulfide bond between α -Cys⁵⁶ and β -Cys³⁴⁰, and expression of a mutant toxin in which β -Cys³⁴⁰ had been replaced by Ser³⁴⁰ resulted in the secretion of a non-toxic α/β heterodimer that is blocked in retrograde transport and incapable of entering the yeast cell cytosol, indicating that one important *in vivo* function of β -Cys³⁴⁰ might be to ensure accessibility of the toxin's β subunit C terminus to the HDEL receptor of the target cell.

Keywords: Kex2p endopeptidase, preprotoxin processing, C-terminal HDEL motif

INTRODUCTION

K28 toxin encoding dsRNA viruses of the yeast *Saccharomyces cerevisiae* contain the genetic information for a secreted non-glycosylated protein toxin and an as yet unidentified immunity component that renders killer cells immune to their own toxin (Tipper & Schmitt, 1991; Wickner, 1992). The secreted and biologically active toxin is a disulfide-bonded heterodimer consisting of a 10.5 kDa α subunit and a 10.9 kDa β subunit (referred to hereafter as α and β) (Schmitt & Tipper, 1995) that kills sensitive non-killer cells by a rapid inhibition of DNA synthesis. Toxin-treated cells arrest in early S phase of the cell cycle with a medium-sized

bud, a single nucleus in the mother cell and a pre-replicated DNA content (Schmitt *et al.*, 1989, 1996). Sequence analysis of a K28 toxin-encoding cDNA predicts that the toxin is matured from a 38 kDa pre-protein precursor corresponding to the 38 kDa preprotoxin (pptox) seen after *in vitro* translation of the toxin-encoding virus transcript (Schmitt, 1995).

Based on the pptox sequence we previously predicted that the toxin precursor includes an N-terminal signal sequence [necessary for toxin import into the ER (endoplasmic reticulum) lumen] that is cleaved by the action of signal peptidase (SP) most likely after Leu³¹ or Gly³⁶, producing a 314- or 309-residue precursor, respectively (Schmitt & Tipper, 1995). The protoxin resulting from SP cleavage contains an intervening, potentially N-glycosylated γ sequence that separates the α and β subunits. Genetic analysis of pptox gene

Abbreviations: ER, endoplasmic reticulum; MBA, methylene blue agar; pptox, preprotoxin; SP, signal peptidase.

expression in yeast $\Delta kex1$ and/or $\Delta kex2$ null mutants and determination of the N-terminal sequences of the toxin's α and β subunits implied a pattern of processing that strongly resembles prohormone conversion in mammalian cells (Steiner *et al.*, 1992; Schmitt & Tipper, 1992; Eisfeld *et al.*, 2000). In *S. cerevisiae*, the KEX-encoded prohormone processing machinery is involved not only in the activation of virally encoded killer toxins (such as K1, K2 and K28) but also in the maturation of the yeast pheromone α factor (Dmochowska *et al.*, 1987; Zhu *et al.*, 1987; Dignard *et al.*, 1991; Eisfeld *et al.*, 2000). Both processing enzymes, endopeptidase Kex2p and carboxypeptidase Kex1p, are membrane-anchored proteins located in a late Golgi compartment and have their N-terminal active sites in the lumen (Fuller *et al.*, 1989; Redding *et al.*, 1991). In both mammalian and yeast systems, endopeptidase Kex2p and Kex2p-like convertases such as furin and PC1-PC7 are very similar in their substrate specificity for C-terminal lysine and arginine residues, most often cutting after a sequence of two basic residues (Bryant & Boyd, 1993; Bevan *et al.*, 1998). In the case of the K28 toxin precursor, intracellular pptox processing has previously been predicted to be mediated by Kex2p and Kex1p (Schmitt & Tipper, 1995). The N terminus of α should be produced by cleavage after Glu-Arg⁴⁹, 18 residues downstream of the predicted SP cleavage site, and the N terminus of β by Kex2p cleavage after Lys-Arg²⁴⁵. Although the precise C terminus of α has not yet been identified, it has been postulated that it terminates upstream of the first N-glycosylation site (Asn-Ser-Thr¹⁶³) since the secreted toxin is not glycosylated *in vivo*. In contrast to the toxin's α subunit, the β C terminus (His-Asp-Glu-Leu³⁴⁴) is generated by the action of yeast carboxypeptidase Kex1p that removes the terminal arginine residue and finally uncovers the toxin's ER targeting signal (β -HDEL), which has recently been shown to be essential for retrograde toxin transport in a sensitive target cell (Eisfeld *et al.*, 2000).

To extend the *in silico* predictions for pptox processing, we have now performed site-directed mutagenesis of the pptox gene and analysed the phenotypic effects on K28 toxicity, immunity and toxin secretion in wild-type yeasts as well as in yeast mutants defective in protein precursor processing ($\Delta kex2$ and/or $\Delta yps1$). We mapped the signal peptidase cleavage site and identified Kex2p sites that are necessary for the *in vivo* maturation of α and β . Finally, we show that the two subunits are covalently linked by a single disulfide bond between α -Cys⁵⁶ and β -Cys³⁴⁰, and present evidence that the most important *in vivo* function of Cys³⁴⁰ might be to expose the toxin's β C-terminal ER targeting signal, ensuring accessibility of the HDEL receptor of the target cell.

METHODS

Strains and culture conditions. *Escherichia coli* DH5 α [F⁻ *recA1 endA1 gyrA96 hsdR17 supE44 relA* Δ (*argF-lacZYA*) *U169* ϕ 80*dlacZ* Δ M15 λ^-] was used as a general host for the amplification and propagation of all constructed plasmids. The following yeast (*Saccharomyces cerevisiae*) strains were

used throughout this study: MS300c [MAT α *leu2 ura3-52 ski2-2* (K28 killer strain)] (Schmitt & Tipper, 1990); 192.2d (MAT α *ura3 leu2*) (Schmitt *et al.*, 1996); SEY6210 (MAT α *ura3-52 leu2-3,112 his3- Δ 200 trp1- Δ 901 lys2-801 suc2- Δ 9*) (Eisfeld *et al.*, 2000); HKY20-11A (MAT α *can1-100 ade2-101 his3-11,15 leu2-3,112 trp1-1 ura3-1 Δ yap3/yps1::LEU2*) (Fuller *et al.*, 1989); BFY113 (MAT α *ura3 ade2 trp1 leu2 his3 can1 kex2::TRP1*); W303-1B (MAT α *ura3 ade2 trp1 leu2 his3 can1*); and ME938 (MAT α *bar1 leu2-3,112 gal2 ura3 his3 kex2::ura3 yap3/yps1::LEU2*) (Egel-Mitani *et al.*, 1990). All yeast cultures were grown at 30 °C either in complex YEPD medium or in synthetic medium (YNB) supplemented with the appropriate amino acid/base requirements of each strain.

DNA constructs and *in vitro* mutagenesis. The K28 killer toxin ORF was isolated by PCR using *Taq* DNA polymerase and the yeast expression plasmid pPGK-M28-1 (Schmitt, 1995) as DNA template. Oligonucleotide primers used for PCR amplification were 5'-CCGGAATTCATGGAGAGCGTTT-CCTCATTATTT-3' and 5'-CCCAAGCTTTTAGCGTAG-CTCATCGTGGCACCT-3', introducing a 5' terminal *Eco*RI and a 3' terminal *Hind*III restriction site flanking the toxin-encoding cDNA. The PCR product containing the K28 ORF was cloned between the *Eco*RI and *Hind*III restriction sites of yeast multicopy vector pYX242, allowing constitutive expression of a K28 killer phenotype under transcriptional control of the yeast triose phosphate isomerase (*TPI*) promoter. The Arg $\rightarrow$ Ala⁴⁹ mutation was introduced by PCR (splicing by overlapping extension) as previously described by Horton *et al.* (1989) using the following primers: 5'-K28, 5'-GAATTCATGGAGAGCGTTTCTCATTATTTAACA-TTTTTTC-3'; 3'-K28, 5'-CTCGAGTTAGCGTAGCTCA-TCGTGGCACCTTG-3'; 5'-R49A, 5'-ATCTGAGAGACA-GCAGGGCTTAGAAGAAGCTGACTTCAGTGCTGCT-ACTTGCGTACT-3' and 3'-R49A, 5'-AGTACGCAAGTAG-CAGCACTGAAGTCAGCTTCTTCTAAGCCCTGCTG-TCTCTCAGAT-3'. For changing Arg¹⁴⁹ into Ala¹⁴⁹, primers 5'-K28, 3'-K28 (see above), 5'-R149A (5'-GACTGAAACG-CAGAGAATATACAATCGGCGAGTCTTATACCGGG-TCTGCTTAGTAGGGATATAA-3') and 3'-R149A (5'-TT-ATAATCCATACTAAGCAGACCCGGTATAAGACTA-GCCGATTGTATATTCTCTGCGTTTTTCAGTC-3') were used. Both constructs were cloned between the *Eco*RI and *Xho*I site of pYX242. The double mutant (Ala⁴⁹/Ala¹⁴⁹) was constructed by substitution of the *Bam*HI-*Xho*I fragment of Arg $\rightarrow$ Ala⁴⁹ by the corresponding fragment of Arg $\rightarrow$ Ala¹⁴⁹. All other amino acid changes were introduced into the K28 ORF by using the phagemid *in vitro* mutagenesis kit Muta-Gene Version 2 (Bio-Rad) under conditions recommended by the manufacturer. The sequences of the oligonucleotide primers used to generate mutations within the N-terminal hydrophobic region of pptox were 5'-ATGTCGGCATTTCGCCG-TGCATATT-3', leading to a change of Gly³⁶ to arginine, and 5'-CGTGCATATTTGGGATTTGAAACA-3' for the exchange of Leu³¹ to proline. The oligonucleotide primer 5'-ATAAGACTACGCTTTTGATATTTTC-3' was used to exchange serine-148 into lysine, thereby conferring the endopeptidase cleavage site predicted to generate the α C terminus into a 'classical' Kex2p site. For an exchange of Cys⁵⁶ to tryptophan the primer 5'-GCCCATCAGTACCCAAGTAG-CAGCA-3' was used. PCR mutagenesis was carried out to change Cys³⁴⁰ to serine with the following oligonucleotides as PCR primers: 5'-CCCAAGCTTTTAGCGTAGCTCATCG-TGGGACCTTGCCTCGTCGTC-3' and 5'-CCGGAATTC-ATGGAGAGCGTTTCTCATTATTT-3'. Mutated pptox constructs were cloned between the *Eco*RI and *Hind*III sites of the yeast expression vector pYX242. Subsequent DNA se-

quencing of the PCR-amplified K28 pptox fragment identified two bases that always differed from the published K28 sequence (Schmitt & Tipper, 1995). According to the base positions in the published K28 cDNA sequence, cytosine in position 779 has to be changed into a thymine, and adenine in nucleotide position 981 has to be changed into a thymine. The latter changes codon 256 from the amino acid serine into phenylalanine. Moreover, due to PCR amplification of the wild-type K28 ORF (prior to *in vitro* mutagenesis), an additional codon change has occurred leading to a change of Arg³²⁸ to glycine. However, the strength of the killer phenotype of transformants expressing the killer toxin with the altered sequence was comparable to transformants expressing the wild-type ORF.

Transformation and DNA sequencing. Wild-type and mutated pptox plasmids were transformed into the indicated yeast strains by the lithium acetate method using salmon sperm DNA as carrier (Schiestl & Gietz, 1989). Where indicated, strains BFY113, ME938 and HKY20-11A were co-transformed with YEp6-KEX2 (Zhu *et al.*, 1992) and maintained on SC-Ura-His medium. YEp6-KEX2 is a 2 μ multi-copy plasmid that carries the *HIS3* gene and a copy of *KEX2* lacking the cytoplasmic C-terminal domain (Zhu *et al.*, 1992). *E. coli* strain DH5 α was transformed by electroporation using the Gene Pulser II system (Bio-Rad). All DNA constructs used in this work were routinely sequenced by fluorescent cycle sequencing on an automated DNA sequencer (LI-COR 4200, MWG Biotech).

Killer assay and immunity tests. K28-specific killing and immunity was determined in an agar diffusion assay on methylene-blue agar plates (MBA; pH 4.7) as previously described (Schmitt & Tipper, 1990). We used citrate at a final concentration of 2.9% instead of 1.92% as described in the original protocol. Killer strains (about 10⁵ cells in a total volume of 10 μ l) were spotted onto MBA plates that had been seeded with an overlay of the sensitive strain *S. cerevisiae* 192.2d (Schmitt *et al.*, 1996). After incubating the plates for 4 days at 20 °C, clear zones of growth inhibition surrounding the killer cells (which indicates toxicity) were measured. Killer activity is expressed as the size of the growth inhibition zone. Toxin sensitivity/immunity tests were performed on MBA plates using an overlay of the indicated yeast strain (10⁵ cells per plate) and concentrated culture supernatants of a K28 killer strain as the toxin source. Briefly, concentrated culture supernatants (100 μ l) derived from the K28 *ski2* mutant *S. cerevisiae* MS300c (Schmitt & Tipper, 1990) were pipetted into wells (9 mm diameter) that had been cut into the agar and the plates incubated for 4 days at 20 °C. In this assay, yeast transformants expressing a mutant pptox derivative that is incapable of conferring functional K28 immunity show a sensitive non-killer phenotype, resulting in a cell-free growth inhibition zone around the well.

Yeast cell fractionation. For subcellular toxin localization, the sensitive yeast strain *S. cerevisiae* 192.2d was grown at 30 °C in YEPD medium to early exponential phase (1 $\times$ 10⁷ cells ml⁻¹), harvested by centrifugation and used for cell fractionation experiments essentially as previously described (Eisfeld *et al.*, 2000). Briefly, yeast spheroplasts were incubated for 1 h at 30 °C in the presence of 10⁴ U purified K28 toxin ml⁻¹. Thereafter, cells were washed in a solution of 0.8 M sorbitol, 20 mM HEPES/KOH, 50 mM potassium acetate and 2 mM EDTA (pH 7.0) and lysed in chilled lysis buffer with the aid of a 5 ml dounce homogenizer (~15 strokes), and the resulting lysate was subjected to differential centrifugation giving four different cell fractions: cell wall

fraction (300 g), membrane fraction (plasma-, Golgi- and ER membranes; 13000 g), endosomal vesicle fraction (100000 g pellet) and cytosol (100000 g supernatant).

Western blot analysis. To estimate the amount of killer toxin secreted by *S. cerevisiae* after transformation with the various K28 expression vectors, cultures of the appropriate transformants were grown in synthetic minimal medium (pH 4.7) at 30 °C for 2 days until they reached about 5 $\times$ 10⁷ cells ml⁻¹. After centrifugation, 1.2 ml supernatant was ethanol precipitated. The precipitate was dried and redissolved in water for further analysis by SDS-PAGE. If not otherwise stated, SDS-PAGE was performed under non-reducing conditions. Reducing conditions were provided by adding β -mercaptoethanol to the sample buffer at a final concentration of 6%. Cell lysis was carried out under reducing conditions using 10⁸ cells which were vortexed for 1 min in sample buffer containing an equal volume of acid-washed glass beads. After short-spin centrifugation, the resulting supernatant was analysed by gel electrophoresis. Samples were fractionated either on SDS-polyacrylamide gradient gels (10–22.5%) or on Tris/Tricine gels (10%) and electrophoretically blotted onto PVDF membranes in transblot buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.1% SDS). Western blot analysis of the secreted toxin was carried out using polyclonal antibodies against the toxin's α and/or β subunit. Experiments on yeast cell fractionation and subcellular toxin localization were performed as previously described (Eisfeld *et al.*, 2000).

Protein sequencing. The secreted pptox processing intermediate in which the two Kex2p sites flanking the N and C termini of α had been destroyed (Arg $\rightarrow$ Ala⁴⁹, Arg $\rightarrow$ Ala¹⁴⁹) was fractionated by SDS-PAGE, electroblotted onto PVDF membranes and used for N-terminal sequence analysis by Edman degradation as previously described (Schmitt & Tipper, 1995).

RESULTS

Identification of the SP cleavage site

In vivo processing of the virally encoded K28 toxin precursor results in the secretion of a disulfide-bonded, heterodimeric protein toxin consisting of a 10.5 kDa α subunit and a 10.9 kDa β subunit. We previously reported that the N terminus of the unprocessed toxin precursor contains two consecutive hydrophobic stretches that are consistent with secretion signal function (Schmitt & Tipper, 1995). However, a plot of SP cleavage probability according to the rules codified by von Heijne (1986) identified no clearly defined signal and this apparent redundancy in signal function predicted SP cleavage sites after either Leu³¹ or Gly³⁶. To identify the precise SP cleavage site, we used site-directed mutagenesis and changed the wild-type sequence (Leu³¹) into proline (Leu $\rightarrow$ Pro³¹) and, in addition, changed Gly³⁶ into arginine (Gly $\rightarrow$ Arg³⁶). Each mutated pptox construct was transformed into the sensitive non-killer strain *S. cerevisiae* SEY6210 and constitutively expressed under transcriptional control of the yeast *TPI* promoter. Transformants were isolated and subsequently tested for killer phenotype expression by determining K28 toxicity, functional immunity and level of toxin secretion. As expected, yeast cells expressing the wild-type pptox gene showed an immune killer phenotype as did

Fig. 1. Destruction of the SP cleavage site within the K28 toxin precursor prevents Kex2p-mediated processing and blocks toxin secretion. The indicated wild-type and mutated pptox derivatives were expressed in the yeast *S. cerevisiae* SEY6210 and their phenotypic effects on K28 toxicity and toxin secretion were determined. (a) Agar diffusion bioassay to determine the *in vivo* toxicity of yeast transformants on MBA (pH 4.7) against the sensitive *S. cerevisiae* tester strain 192.2d. (b) Western blot analysis of K28 toxin secreted by yeast cells expressing the wild-type pptox and two mutated pptox derivatives carrying substitutions in the predicted SP cleavage sites. (c) Immunoblot analysis of cell extracts enriched for endosomal and/or secretory vesicles derived from yeasts expressing either the wild-type pptox or the mutated pptox variant Gly→Arg³⁶. (b, c) Samples were separated by SDS-PAGE under non-reducing conditions (b) and reducing conditions in the presence of β -mercaptoethanol (c) and probed with a polyclonal antibody against β . Arrows indicate position and size (in kDa) of the heterodimeric protein toxin (α/β) and of its β subunit.

transformants expressing the Leu→Pro³¹ pptox derivative (Fig. 1a). Western analysis of cell-free culture supernatants further indicated that toxin secretion was not negatively affected in the Leu→Pro³¹ mutant and rather resembled the wild-type level (Fig. 1b). In contrast, yeast transformants expressing the Gly→Arg³⁶ toxin variant were phenotypically sensitive non-killers which failed to show any toxin secretion (Fig. 1) and, in addition, were unable to express functional K28 immunity against the exogenously applied virus toxin (data not shown). The observed lack of toxin secretion indicates that SP cleavage is prevented in the Gly→Arg³⁶ toxin derivative. Furthermore, Western analysis of cell extracts from yeasts expressing the mutated pptox (Gly→Arg³⁶) gave no signal for the fully processed and matured β , while the same extract derived from cells expressing the wild-type toxin gave a clearly detectable β signal within the endosomal and/or secretory vesicle fraction (Fig. 1c). Since the mutated pptox was primarily detectable as a low-molecular-mass protein smear within the yeast cell cytosol (data not shown), it can be predicted that a Gly→Arg³⁶ pptox variant is retranslocated from the ER into the cytosol where it is probably targeted for proteasomal degradation. As indicated below, N-terminal sequence analysis of a secreted K28 processing intermediate in which both Kex2p sites flanking α had been destroyed (Arg→Ala⁴⁹/Arg→Ala¹⁴⁹) produced the amino acid sequence NH₂-Met³⁷-Pro-Thr-Ser-Glu-Arg-Gln-Gln-Gly-Leu, which matched exactly the N terminus of the unprocessed toxin precursor (δ - α - γ - β) as predicted from the pptox cDNA sequence (Schmitt & Tipper, 1995). SP cleavage after Gly³⁶ is thereby confirmed (see also Fig. 3a).

Involvement of Kex2p in K28 pptox processing

N-terminal amino acid sequence analysis of the purified toxin and comparison with the cDNA sequence of the unprocessed toxin precursor indicated that α and β are generated *in vivo* by endopeptidase cleavage, initiating

at Asn⁵⁰ and Ala²⁴⁶ respectively (Schmitt & Tipper, 1995). *In vitro* analysis of Kex2p endopeptidase activity against different peptides showed that Kex2p cleavage occurs most efficiently after Lys-Arg, Arg-Arg, and (with much lower efficiency) after Pro-Arg and Ala-Arg (Brenner & Fuller, 1992). *In vivo*, the context would be expected to affect cleavage efficiency, and inspection of known Kex2p processing sites indicates preference for sites with a hydrophobic residue in the P4 position (Tao *et al.*, 1990; Park *et al.*, 1994). Following these rules, Kex2p cleavage within the K28 precursor should occur after Leu-Tyr-Lys-Arg¹⁹² (within γ) and after Leu-Gln-Lys-Arg²⁴⁵ (generating the mature β N terminus). Besides that, the K28 toxin precursor contains no other clearly predictable Kex2p cleavage sites.

By classical genetics and phenotypic analysis we previously reported that pptox expression in a yeast Δ kex2 null mutant results in immune non-killers completely devoid of secreted toxin activity or protein (Schmitt & Tipper, 1995). It, therefore, had been concluded that KEX2 function is essential for expression and secretion of the α/β heterodimeric protein toxin. We now extend these findings by analysing the intracellular K28 processing intermediates in a yeast Δ kex2 null mutant. Expression of wild-type pptox in this mutant (strain BFY113) resulted in immune non-killers that did not secrete detectable amounts of toxin (Fig. 2a, lane 2). However, in contrast to the cell-free culture supernatant, intracellular membrane fractions derived from the Δ kex2 mutant gave a strong anti- β antibody response and identified a 42 kDa protein species which was absent in the negative control (i.e. in the untransformed mutant; Fig. 2b, lanes 4 and 5, respectively). Thus, in a Δ kex2 mutant, the K28 toxin precursor is accumulating within the intracellular membrane fraction – most likely within the ER lumen – because it is not properly processed. Correspondingly, retransformation of the pptox-expressing Δ kex2 mutant with a functional copy of KEX2 on the 2 μ vector YEp6-KEX2 fully restored pptox processing, resulting in immune killers that

Fig. 2. Yeast $\Delta kex2$ mutants are blocked in pptox processing and toxin secretion. Immunoblot of proteins produced by a pptox expressing yeast $\Delta kex2$ mutant before and after co-transformation with YEp6-KEX2. Proteins of the cell-free culture supernatant and of the intracellular membrane fraction of the K28 pptox expressing $\Delta kex2$ mutant BFY113 were separated by SDS-PAGE under reducing conditions in the presence of β -mercaptoethanol and detected using anti- β polyclonal rabbit antiserum. Where indicated, the $\Delta kex2$ null mutant was co-transformed with YEp6-KEX2, which is a 2 μ plasmid carrying a copy of *KEX2*. (a) Reducing SDS-PAGE and Western analysis of the secreted toxin (β subunit) in cell-free culture supernatants of the $\Delta kex2$ mutant BFY113 before and after transformation with YEp6-KEX2. Lanes: 1, culture supernatant of the toxin-secreting killer strain MS300c (positive control); 2, culture supernatant (10-fold concentrated) of the pptox expressing $\Delta kex2$ mutant BFY113; 3, as lane 2 but after co-transformation with YEp6-KEX2; M, prestained marker proteins. (b) Proteins of the intracellular membrane fraction of the $\Delta kex2$ mutant BFY113 transformed with the K28 pptox expression vector pPGK-M28-I. Lane 4, intracellular membrane fraction of $\Delta kex2$ mutant cells accumulating the unprocessed 42 kDa toxin precursor (protoxin); lane 5, intracellular membrane fraction of the same mutant without pPGK-M28-I (negative control).

secreted significant amounts of a biologically active α/β toxin (Fig. 2a, lane 3; see also Table 1). Interestingly, the 42 kDa protein species seen within the intracellular membrane fraction of a $\Delta kex2$ mutant perfectly matches the predicted size of the 37.6 kDa toxin precursor after entry into the ER and subsequent core-glycosylation within γ : once the toxin precursor has entered the ER lumen, pptox processing should initiate with SP cleavage

after Gly³⁶ (see above) and further N-glycosylation should add about 7.5 kDa leading to a protoxin with a calculated size of 41.9 kDa.

Kex2p-mediated processing of the toxin's α N and C termini

Since SP cleavage of the K28 pptox precursor occurs after Gly³⁶, and the α N terminus in the mature toxin initiates at Asp⁵⁰, additional N-terminal processing is needed to produce the mature N terminus of the toxin's α subunit. Furthermore, α is not glycosylated *in vivo* and, therefore, it can be predicted that it terminates before the first N-glycosylation site within γ (Asn-Ser-Thr¹⁶³), whose context suggests facile N-glycosylation. Possible endopeptidase cleavage sites responsible for generating the mature α N and C termini are after Leu-Glu-Glu-Arg⁴⁹ (α N) and Ile-Gln-Ser-Arg¹⁴⁹ (α C), although neither sequence resembles the established Kex2p specificity profile. In yeast, Yps1p (formerly called Yap3p) is another membrane-anchored aspartyl protease of the yapsin family, which has been shown to be responsible for presomatostatin cleavage after Leu-Glu-Arg in recombinant somatostatin expressing yeast cells (Bourbonnais *et al.*, 1993). Furthermore, over-expression of YAP3/YPS1 in a $\Delta kex2$ null mutant can partially suppress loss of Kex2p activity (Egel-Mitani *et al.*, 1990), indicating some overlap in cleavage specificity between Kex2p and Yps1p. Interestingly, the predicted endopeptidase cleavage site upstream of the toxin's α N terminus (Leu-Glu-Glu-Arg⁴⁹) shows striking similarity to the sequence Leu-Glu-Arg recognized during presomatostatin processing in yeast, and the yapsin Yps1p might indeed play a role in the maturation of the α N terminus. To address this question we destroyed the potential Kex2p sites at both termini of α by changing the P1 position of the wild-type sequence into (i) Leu-Glu-Glu-Ala⁴⁹ and (ii) Ile-Gln-Ser-Ala¹⁴⁹ (Fig. 3a). In addition to the single mutants, we also constructed a double mutant (Ala⁴⁹/Ala¹⁴⁹) in which the arginine residue in the P1 position of both α -flanking Kex2p sites was changed into alanine. Wild-type and mutated pptox constructs were expressed in a *KEX2 YPS1* wild-type yeast, in a $\Delta kex2$ null mutant and in a $\Delta yps1$ deletion mutant (strain HKY20-11A), and subsequently analysed for killer phenotype expression and toxin secretion. SDS-PAGE and Western analysis of the cell-free culture supernatants indicated that Kex2⁺ wild-type yeasts (as well as $\Delta yps1$ mutant cells; data not shown) expressing the mutated Ala⁴⁹ toxin derivative secrete a heterodimeric 23.5 kDa protein that – after separation by reducing SDS-PAGE – dissociated into a 12.3 kDa subunit (resembling the unprocessed δ/α fragment) and the correctly processed 10.9 kDa β subunit (Fig. 3b, right lane in each blot). *In vivo* expression of the Ala¹⁴⁹ derivative resulted in the secretion of a 32 kDa heterodimer consisting of a 21.1 kDa α/γ fragment and (again) the fully processed 10.9 kDa β subunit (Fig. 3b, middle lane in each blot). Correspondingly, expression of the Ala⁴⁹/Ala¹⁴⁹ double mutant resulted in the secretion of a 35 kDa protein that, after separation by reducing SDS-

Table 1. Pptox processing in yeast $\Delta kex2$ null and $\Delta kex2 \Delta yps1$ double null mutants

Killer phenotype and toxin secretion in wild-type and in mutant yeasts defective in Kex2p- and/or Yps1p-mediated precursor processing after expression of wild-type and various mutated pptox variants before and after co-transformation with YEp6-KEX2. NT, Not tested.

Yeast strain (relevant genotype)	Expressed pptox variant	Phenotype	Phenotype after cotransformation with YEp6-KEX2	Secreted toxin fragments* after cotransformation with YEp6-KEX2
SEY6210 (<i>KEX2 YPS1</i>)	Wild-type	Killer	NT	$\alpha + \beta$
	Arg→Ala ⁴⁹	Non-killer	NT	$\delta/\alpha + \beta$
	Arg→Ala ¹⁴⁹	Non-killer	NT	$\alpha/\gamma + \beta$
	Ala ⁴⁹ /Ala ¹⁴⁹	Non-killer	NT	$\delta/\alpha/\gamma + \beta$
BFY113 (<i>kex2::TRP1</i>)	Wild-type	Non-killer	Killer	$\alpha + \beta$
	Arg→Ala ⁴⁹	Non-killer	Non-killer	$\delta/\alpha + \beta$
	Arg→Ala ¹⁴⁹	Non-killer	Non-killer	$\alpha/\gamma + \beta$
	Ala ⁴⁹ /Ala ¹⁴⁹	Non-killer	Non-killer	$\delta/\alpha/\gamma + \beta$
ME938 ($\Delta kex2 \Delta yps1$)	Wild-type	Non-killer	Killer	$\alpha + \beta$
	Arg→Ala ⁴⁹	Non-killer	Non-killer	$\delta/\alpha + \beta$
	Arg→Ala ¹⁴⁹	Non-killer	Non-killer	$\alpha/\gamma + \beta$
	Ala ⁴⁹ /Ala ¹⁴⁹	Non-killer	Non-killer	$\delta/\alpha/\gamma + \beta$
HKY20-11A ($\Delta yps1$)	Wild-type	Killer	Killer	$\alpha + \beta$
	Arg→Ala ⁴⁹	Non-killer	Non-killer	$\delta/\alpha + \beta$
	Arg→Ala ¹⁴⁹	Non-killer	Non-killer	$\alpha/\gamma + \beta$
	Ala ⁴⁹ /Ala ¹⁴⁹	Non-killer	Non-killer	$\delta/\alpha/\gamma + \beta$

* Cell-free culture supernatants from the indicated yeast transformants were concentrated by ethanol precipitation, separated by SDS-PAGE under reducing conditions in the presence of β -mercaptoethanol and probed with a polyclonal antibody against the toxin's α and/or β subunit (see also legend to Fig. 3). The toxin fragments identified by Western analysis had the following sizes: 10.5 kDa (α), 10.9 kDa (β), 12.2 kDa (δ/α), 21.1 kDa (α/γ) and 22.6 kDa ($\delta/\alpha/\gamma$).

PAGE in the presence of β -mercaptoethanol, dissociated into its two components: a 22.6 kDa $\delta/\alpha/\gamma$ fragment and a 10.9 kDa β subunit (Fig. 3b, left lane in each blot). Since expression of the mutated pptox constructs always resulted in a non-killer phenotype independent of the host cell's genotype and, furthermore, since processing of wild-type pptox was equally effective in a *KEX2 YPS1* wild-type and in a $\Delta yps1$ null mutant (Table 1), it can be concluded that Yps1p is not involved in pptox processing *in vivo*. Interestingly, changing the wild-type pptox sequence Ile-Gln-Ser-Arg¹⁴⁹ into Ile-Gln-Lys-Arg¹⁴⁹ and thus converting the wild-type sequence into a 'classical' Kex2p endopeptidase cleavage site enhanced neither K28 toxicity nor toxin secretion (data not shown), indicating that Kex2p cleavage after Ser-Arg¹⁴⁹ occurs equally effectively as after Lys-Arg¹⁴⁹. Based on these data we conclude that in the wild-type toxin precursor Leu-Glu-Glu-Arg⁴⁹ and Ile-Gln-Ser-Arg¹⁴⁹ represent amino acid sequences that are effectively cleaved by Kex2p *in vivo*.

Investigation of potential Kex2p processing sites within γ and at the N terminus of β

For the potential Kex2p site within γ we could show that this sequence (Leu-Tyr-Lys-Arg¹⁹²) is probably not cleaved *in vivo* because (as shown above) expression of an Arg→Ala¹⁴⁹ toxin derivative resulted in the secretion

of a α/γ fragment that corresponded exactly to the predicted size of the unprocessed α/γ peptide after translocation into the ER, cleavage of the signal peptide and core N-glycosylation of all three potential N-glycosylation sites (Asn-X-Ser/Thr) within γ . However, the observed molecular mass of the secreted pptox processing intermediates indicates that the N-linked carbohydrate moiety of all γ -containing fragments is probably trimmed back by late Golgi α -mannosidases.

As summarized in Table 1, exactly the same results were obtained after expression of the Arg→Ala¹⁴⁹ pptox either in the $\Delta kex2$ null mutant BFY113 or in the $\Delta yps1$ mutant HKY20-11A, and no changes were observed after co-expression of Kex2p from YEp6-KEX2 (Table 1): in each case, besides the predicted and identified γ -containing fragments (α/γ , 21 kDa; $\delta/\alpha/\gamma$, 22.6 kDa), no additional α/γ -specific signal was detectable, suggesting that Kex2p does not recognize the internal endopeptidase cleavage site Leu-Tyr-Lys-Arg¹⁹² within γ . If Kex2p cleavage after Lys-Arg¹⁹² did occur *in vivo*, a α/γ fragment of about 15.5 kDa would have been expected; however, no such signal was detectable (see also Fig. 3b). In addition, *in vivo* expression of a mutant pptox in which the Kex2p cleavage site within γ (Lys-Arg¹⁹²) had been destroyed in its P1 position and changed into Lys-Ala¹⁹² resulted in a normal killer phenotype indistinguishable from that of yeast transformants expressing the wild-type pptox (data not shown).

Fig. 3. Schematic structure of mutated pptox derivatives and their phenotypic effects on K28 precursor processing and toxin secretion. (a) The K28 toxin precursor is shown consisting of the secretion leader sequence (aa 1–36) and its SP cleavage site (Gly³⁶), and the four distinct segments δ (aa 37–49), α (aa 50–148), γ (aa 150–243) and β (aa 246–344). The N-terminal sequence determined for a secreted δ - α - γ - β pptox processing intermediate initiating at Met³⁷ is indicated. Predicted sizes (in kDa) of pptox processing intermediates secreted after *in vivo* expression of pptox derivatives in which the two α -flanking Kex2p sites had been destroyed are indicated. (b) Western analysis of secreted K28 processing intermediates present in cell-free culture supernatants of yeasts expressing the indicated pptox derivatives Arg $\rightarrow$ Ala⁴⁹, Arg $\rightarrow$ Ala¹⁴⁹ and the double mutant Ala⁴⁹/Ala¹⁴⁹. In each case, secreted toxin from 1×10^8 cells of *S. cerevisiae* SEY6210 expressing the indicated pptox construct was separated by SDS-PAGE under non-reducing as well as under reducing conditions [in the absence or presence of β -mercaptoethanol (β -ME)] and probed with a polyclonal antibody against the toxin's α and/or β subunit. Arrows indicate the positions of the secreted preprotoxin processing intermediates $\delta\alpha\gamma\beta$, $\alpha\gamma\beta$ and $\delta\alpha\beta$. The protein band marked with an asterisk (*) represents the 21.1 kDa $\alpha\gamma$ fragment resulting from spontaneous reduction of the disulfide bonded $\alpha\gamma/\beta$ -heterodimeric protein toxin.

Since the endopeptidase cleavage site immediately upstream of the mature β N terminus already resembles a 'classical' Kex2p site (Leu-Gln-Lys-Arg²⁴⁵), we asked if a change in the P2 position (Leu-Gln-Gln-Arg²⁴⁵) prevents or weakens Kex2p cleavage activity at this site. *In vivo* expression of the mutated Gln-Arg²⁴⁵ pptox construct resulted in immune non-killers that were significantly inhibited in toxin secretion. While no detectable signal for a correctly processed α/β toxin was seen in immunoblots of cell-free culture supernatants, Western analysis of subcellular fractions enriched for endosomal and/or secretory vesicles indicated that Gln-Arg²⁴⁵ expressing yeasts are able to produce and secrete traces of a correctly processed 10.9 kDa β subunit (Fig. 4). The low amounts of β seen within the secretory vesicle fraction of Gln-Arg²⁴⁵ expressing cells might be due to the residual capacity of Kex2p to partially process the mutated toxin precursor, since the same result was obtained in a yeast $\Delta yps1$ mutant, while no such signal

was detectable in the $\Delta kex2$ mutant strain BFY113 (data not shown).

Disulfide bond formation between Cys⁵⁶ and Cys³⁴⁰ exposes the toxin's β -C-terminal ER targeting signal and ensures retrograde toxin transport *in vivo*

Since the M28 cDNA sequence identified a single cysteine residue at amino acid position 7 of the mature α toxin (resembling Cys⁵⁶ in the unprocessed pptox sequence), Cys⁵⁶ must be involved in the disulfide bonding of the α/β heterodimeric protein toxin. In contrast, β contains four cysteine residues (Cys²⁹², Cys³⁰⁷, Cys³³³ and Cys³⁴⁰) and it is not predictable which cysteine residue constitutes the intermolecular disulfide bond between α and β in the mature toxin. Since we recently demonstrated that the C-terminal HDEL motif in β is essential for *in vivo* K28 toxicity by ensuring retrograde transport of the α/β toxin (Eisfeld *et al.*,

Fig. 4. Destruction of the Kex2p cleavage site upstream of β prevents pptox processing and toxin secretion. SDS-PAGE and immunoblot analysis of cell extracts enriched for secretory vesicles derived from yeasts expressing the wild-type pptox gene (Leu-Gln-Lys-Arg²⁴⁵) or a mutated derivative in which the potential Kex2p site predicted to generate the mature β N terminus had been destroyed (Leu-Gln-Gln-Arg²⁴⁵). Protein samples were separated by SDS-PAGE under reducing conditions in the presence of β -mercaptoethanol, blotted onto a PVDF membrane and probed with a polyclonal anti- β antibody. M, markers.

2000), we speculated that the cysteine residue right next to the β C-terminal HDEL motif (Cys³⁴⁰) might have the important *in vivo* function of exposing the toxin's ER targeting signal to the HDEL receptor of a sensitive yeast cell. To address this question, we used site-directed mutagenesis and destroyed the only cysteine residue in α (Cys⁵⁶) as well as the C-terminal cysteine residue in β (Cys³⁴⁰). As shown in Fig. 5a, changing cysteine-56 into tryptophan (Cys $\rightarrow$ Trp⁵⁶) and subsequent expression of the mutated pptox construct in yeast resulted in immune non-killers unable to secrete detectable amounts of toxin. In contrast to the observed lack of toxin secretion after *in vivo* expression of the Cys $\rightarrow$ Trp⁵⁶ toxin variant, expression of toxin derivatives in which any of the three cysteine residues Cys²⁹², Cys³⁰⁷ or Cys³³³ in β had been changed into tryptophan always resulted in toxin-secreting yeasts that showed a normal wild-type killer phenotype (data not shown). It can therefore be concluded that in the wild-type toxin cysteine residues Cys²⁹², Cys³⁰⁷ and Cys³³³ are not involved in disulfide bond formation *in vivo*. Interestingly, a mutated toxin derivative in which the cysteine residue closest to the β C-terminal HDEL signal had been changed into serine (Cys $\rightarrow$ Ser³⁴⁰) still resulted in the secretion of an α/β heterodimeric protein, but the biological activity of such a toxin was completely lost (Fig. 5a, b). Although secretion of the mutant Cys $\rightarrow$ Ser³⁴⁰ toxin was slightly decreased compared to the wild-type (Fig. 5a; compare left and right lanes), the stability and electrophoretic mobility of the mutant protein exactly portrayed the behaviour of the wild-type protein toxin. Just like the wild-type toxin, the mutant Cys $\rightarrow$ Ser³⁴⁰ toxin dissociates into its two subunits α and β under conditions of a

Fig. 5. Disulfide bond formation between Cys⁵⁶ (α) and Cys³⁴⁰ (β) is essential for K28 toxicity, toxin secretion and retrograde toxin transport. Mutated pptox derivatives in which the only cysteine residue in α had been destroyed (Cys $\rightarrow$ Trp⁵⁶) or in which the β -C-terminal cysteine residue immediately upstream of the toxin's ER targeting signal had been changed into a serine (Cys $\rightarrow$ Ser³⁴⁰) were expressed in the sensitive non-killer yeast *S. cerevisiae* SEY6210 and tested for killer phenotype expression, toxin secretion and retrograde toxin transport. (a) Toxin secretion in yeast cells expressing the wild-type toxin or the indicated pptox derivatives was determined by SDS-PAGE under non-reducing conditions and Western blot was probed with a polyclonal anti- β antibody (see legend to Fig. 2). (b) Killer phenotype expression was determined in a standard agar diffusion assay on MBA (pH 4.7) as described in the legend to Fig. 1. (c) Spheroplasts of the sensitive strain *S. cerevisiae* 192.2d were treated either with the wild-type toxin (β -C³⁴⁰HDEL) or with a toxin derivative in which the β -C-terminal cysteine residue had been destroyed (β -S³⁴⁰HDEL). After osmotic cell lysis and differential centrifugation, the resulting cell fractions were separated by SDS-PAGE and the Western blot was probed with a polyclonal anti- β antibody. M, intracellular membrane fraction (cytoplasmic, ER and Golgi membranes); E, endosomal vesicle fraction; C, cytosol. Arrows indicate the positions of the heterodimeric toxin (α/β) and of its tetrameric derivative (α/β)₂ that forms spontaneously under conditions of a non-reducing SDS-PAGE. The β signal present within the membrane fraction of yeast cells treated with the wild-type toxin results from spontaneous dissociation of the α/β toxin into its two subunits.

denaturing SDS-PAGE run under reducing conditions (data not shown), strongly indicating that in the mutant toxin an alternative disulfide bond is generated which results in the secretion of a heterodimeric protein whose β C-terminal HDEL motif is 'masked' and no longer

recognizable by the HDEL receptor Erd2p of the target cell. To address this question we treated sensitive yeast cells with the mutated toxin (Cys→Ser³⁴⁰) or with the wild-type toxin and subsequently analysed subcellular fractions for localization of the α/β toxin. Cell fractionation studies of three independently performed experiments always indicated that the mutated toxin is only detectable within the intracellular membrane fraction and not within the yeast cell cytosol, while the wild-type toxin is capable of entering the cytosol (Fig. 5c). These results strongly suggest that the observed loss in toxicity of the Cys→Ser³⁴⁰ mutant toxin is probably caused by its inability to interact with the HDEL receptor Erd2p of the target cell. As a direct consequence, retrograde transport of the mutated Cys→Ser³⁴⁰ toxin is blocked and the toxin can no longer reach its intracellular target. Future experiments will have to demonstrate that the accessibility of the toxin's HDEL signal is actually controlled by disulfide bond formation.

DISCUSSION

SP cleavage and toxin immunity

Until today, three different virally encoded killer toxins have been identified in *S. cerevisiae*. Although all three toxins (K1, K2, K28) show striking similarities with respect to their intracellular processing patterns, they differ significantly in primary structure and mode of action of the mature protein toxins (Dignard *et al.*, 1991; Martinac *et al.*, 1990; Schmitt *et al.*, 1996). Based on the K28 toxin-encoding cDNA sequence we previously postulated that processing of the K28 toxin precursor initiates with SP cleavage and N-glycosylation on entry into the ER (Schmitt & Tipper, 1995). A plot of SP cleavage probability according to the rules of von Heijne (1986) further suggested that the most probable cleavage sites occur after either Leu³¹ or Gly³⁶. By site-directed mutagenesis, *in vivo* expression of the mutated ptox constructs and N-terminal sequencing of a secreted ptox processing intermediate, we now demonstrate that SP cleavage occurs after Gly³⁶. Changing Leu³¹ in the wild-type toxin into a proline had no effect on K28 toxicity, and secretion of a Leu→Pro³¹ toxin derivative resembled the wild-type level. In contrast, *in vivo* expression of a Gly→Trp³⁶ toxin variant resulted in sensitive non-killer yeasts that were completely blocked in toxin secretion. Western analysis of cell extracts derived from yeasts expressing the mutated toxin further indicated that the Gly→Trp³⁶ toxin is detectable as a low-molecular-mass protein smear within the yeast cell cytosol, indicating that the mutated toxin is probably recognized as misfolded protein and targeted for proteasomal degradation. Interestingly, yeast cells expressing the mutated Gly→Trp³⁶ toxin were also defective in expressing functional immunity and were highly sensitive to exogenously applied killer toxin. The observed toxin sensitivity is consistent with earlier studies on K1 immunity in which it was shown that the toxin precursor itself is sufficient to confer immunity as soon as it enters the secretory pathway

(Bostian *et al.*, 1983). Although the molecular basis for toxin immunity is still unknown, it has been speculated that it might be conferred by the toxin precursor itself, in contrast to its components, which can act as competitive inhibitor of the mature toxin by saturating a so far unidentified plasma membrane receptor that normally mediates toxicity (Tipper & Schmitt, 1991; Bostian *et al.*, 1983; Bussey *et al.*, 1982). Within the yeast secretory pathway, the toxin precursor and its membrane receptor are both present long before ptox processing continues in a late Golgi compartment; it, therefore, can be speculated that any mature toxin would not have access to the toxin's plasma membrane receptor, finally resulting in immunity (Sturley *et al.*, 1986; Boone *et al.*, 1986).

Kex2p-mediated ptox processing

Sequence analysis of the K28 toxin precursor did not allow precise predictions on how both termini in α are generated *in vivo*. The most predictable processing sites are after Leu-Glu-Glu-Arg⁴⁹ (generating the α N terminus) and after Ile-Gln-Ser-Arg¹⁴⁹ producing the α C terminus. In each case, this motif is the only basic residue in the vicinity that might mark a cleavage site for known yeast processing endoproteases. Optimal recognition sites for the Kex2p endopeptidase are Lys-Arg or Arg-Arg with preference for sites with a hydrophobic residue in the P4 position (Tao *et al.*, 1990; Park *et al.*, 1994), although it has recently been shown that the α N terminus of the K1 toxin is generated by Kex2p cleavage after Pro-Arg⁴⁴ (Zhu *et al.*, 1992); Glu-Arg⁴⁹ and Ser-Arg¹⁴⁹, however, have not yet been reported as being recognized by Kex2p.

To shed more light onto the K28 precursor processing in yeast, we specifically destroyed/alterd the predicted endopeptidase cleavage sites within the ptox wild-type sequence, expressed both wild-type and mutated ptox genes in a wild-type yeast (Kex2⁺ Yps1⁺), in a Δ kex2 null mutant and in a Δ kex2 Δ yps1 double mutant, and subsequently analysed their phenotypic effects on K28 toxicity and protein secretion. Interestingly, expression of wild-type ptox in a Δ kex2 mutant resulted in a cell-associated 42 kDa protein whose size corresponded to that predicted for an ER precursor after SP cleavage and addition of the three core N-glycosyl groups within γ . Since the unprocessed 42 kDa protoxin was not secreted and was only detectable within the intracellular membrane fraction, it can be speculated that in a Δ kex2 mutant the protoxin is probably retranslocated into the cytosol and targeted for proteasomal degradation.

After *in vivo* expression of mutated ptox derivatives in which either of the two α -flanking Kex2p sites had been destroyed (Ile-Glu-Glu-Ala⁴⁹, Ile-Gln-Ser-Ala¹⁴⁹ and the Ala⁴⁹/Ala¹⁴⁹ double mutant), immune non-killers were obtained that secreted incorrectly matured K28 processing intermediates. In case of the Ala⁴⁹ single mutant and the Ala⁴⁹/Ala¹⁴⁹ double mutant, δ/α - and/or $\delta/\alpha/\gamma$ -containing ptox fragments were secreted whose N

termini (Met³⁷-Pro-Thr-Ser-Glu-Arg-Gln-Gln-Gly-Leu) corresponded exactly to the N terminus of δ after SP cleavage within the ER lumen. Since no other γ -containing toxin fragment was detectable in the cell-free culture supernatant of the corresponding yeast transformant and the same results were obtained after *in vivo* expression of a mutant Lys-Ala¹⁹² toxin derivative, it can be concluded that Kex2p endopeptidase cleavage within γ (Leu-Tyr-Lys-Arg¹⁹²) does not occur *in vivo*. In contrast, a more recent study on K1 toxin precursor processing indicated that the sequence Tyr-Val-Lys-Arg¹⁸⁸ (which is also located within a γ sequence flanking α and β) is cleaved by Kex2p, even though the context does not fit the proposed consensus for Kex2p cleavage (Tao *et al.*, 1990; Park *et al.*, 1994). Interestingly, changing the K28 wild-type sequence Ile-Gln-Ser-Arg¹⁴⁹ into the classical Kex2p cleavage site Ile-Gln-Lys-Arg¹⁴⁹ did not have any effect on killer phenotype expression, and yeast cells expressing such a toxin derivative showed normal killing and were perfectly capable of secreting wild-type levels of a correctly processed α/β heterodimeric protein toxin (data not shown). The possibility that, in the mature α -toxin, both termini are generated by the action of the monobasic endopeptidase Yps1p (Egel-Mitani *et al.*, 1990; Ledgerwood *et al.*, 1996) is highly unlikely since killer phenotype expression in a yeast $\Delta yps1$ null mutant (strain HKY20-11A) was not negatively affected and secretion of a biologically active α/β heterodimeric protein toxin resembled wild-type level (Table 1).

Besides being responsible for the N- and C-terminal processing of α , Kex2p is also involved in the N-terminal processing of the toxin's β subunit, since amino acid sequence analysis of the purified toxin identified that β initiates after Leu-Gln-Lys-Arg²⁴⁵, which represents a classical Kex2p site highly predictable for endopeptidase cleavage *in vivo*. Expression of wild-type ptox in a $\Delta kex2$ null mutant as well as in a $\Delta kex2 \Delta yps1$ double mutant always resulted in non-killer yeasts that were completely blocked in toxin secretion and which accumulated a 42 kDa protoxin within the intracellular membrane fraction. Since in both mutants, activity and secreted protein were fully restored by co-transformation with YEp6-KEX2 (a KEX2 carrying 2 μ vector), we conclude that K28 ptox processing indeed is Kex2p dependent.

A single disulfide bond joins the heterodimeric virus toxin and exposes the toxin's β C-terminal HDEL signal

In the mature heterodimeric protein toxin, α and β are covalently linked by a single disulfide bond. Since the α subunit possesses only one cysteine residue close to its N terminus, this cysteine residue (Cys⁵⁶) must be involved in disulfide bond formation. Correspondingly, a toxin derivative in which the cysteine residue in α had been destroyed (Cys $\rightarrow$ Trp⁵⁶) was biologically inactive since intermolecular disulfide bond formation was prevented. In contrast to the cytotoxic α subunit, β has four cysteine

residues, each of them being possibly involved in disulfide bond formation. We have chosen Cys³⁴⁰ as the most promising candidate for cysteine mutagenesis because this residue is right next to the toxin's β C-terminal HDEL signal, which has recently been shown to be absolutely required for retrograde toxin transport in a sensitive target cell (Eisfeld *et al.*, 2000). Once the toxin has entered a cell by endocytosis, it travels the secretion pathway in reverse (via Golgi and ER) in order to reach the yeast cell cytosol where the cytotoxic signal is transmitted into the nucleus (Schmitt & Eisfeld, 1999). In this respect the virally encoded K28 yeast toxin resembles certain bacterial and plant toxins which likewise are able to enter and kill a eukaryotic target cell by modifying essential cellular components within the cytosol. For some of these heterodimeric protein toxins (like *Pseudomonas* exotoxin A and yeast K28 virus toxin) it has been shown that C-terminal K/HDEL-like sequences are responsible for intracellular targeting and retrograde transport of the toxins (Eisfeld *et al.*, 2000; Yoshida *et al.*, 1991). Correspondingly, mutations in the C-terminal ER targeting sequence dramatically reduce cytotoxicity because interaction of the toxin's C terminus with the K/HDEL receptor of the target cell is prevented (Chaudhary *et al.*, 1990). In this respect it is also interesting to note that some of these toxins contain disulfide bonds at or near their C termini whose *in vivo* function is predicted to ensure access of the toxin's KDEL/HDEL signal to the K/HDEL-receptor of the corresponding target cell (Pelham *et al.*, 1992). This situation is also true for the K28 toxin since we now show that the cysteine residue right next to the β C terminus (Cys³⁴⁰) is part of the disulfide bond that covalently joins α and β . Interestingly, yeast cells expressing the mutated Cys $\rightarrow$ Ser³⁴⁰ toxin derivative showed normal levels of toxin secretion, and Western analysis of cell-free culture supernatants further indicated that a heterodimeric protein is secreted that consists of a 10.5 kDa α and a 10.9 kDa β subunit, indistinguishable from the two subunits in the Cys³⁴⁰ wild-type toxin. It therefore can be concluded that heterodimer formation in the mutated toxin must be catalysed by an alternative disulfide bond between Cys⁵⁶ (in α) and one of the three remaining non-mutated cysteine residues in β . Phenotypic analysis of yeasts expressing the mutated toxin identified the Cys $\rightarrow$ Ser³⁴⁰ toxin as being completely inactive, incapable of killing a sensitive yeast cell. Additional cell fractionation experiments on sensitive yeasts treated with the mutant Cys $\rightarrow$ Ser³⁴⁰ toxin indicated that the observed loss of toxicity is likely to be caused by its inability to retrograde pass a cell and to successfully reach its intracellular target. In this respect, the phenotype of a Cys $\rightarrow$ Ser³⁴⁰ toxin derivative exactly portrays the phenotype of a truncated toxin in which the β C-terminal HDEL sequence had been deleted; *in vivo* such a toxin (β - Δ HDEL) is likewise no longer capable of entering the secretion pathway of a sensitive target cell (Eisfeld *et al.*, 2000). We therefore postulate that in the wild-type toxin, formation of a disulfide bond between Cys⁵⁶ (α) and Cys³⁴⁰ (β) has the important *in vivo* function of ensuring accessibility of the β C-terminal ER

Fig. 6. Model of Kex2p/Kex1p-mediated ptox processing in yeast. The 345-residue toxin precursor is shown consisting of the four distinct segments δ , α , γ and β (see also legend to Fig. 3). Based on the data obtained in this study, processing sites by SP after Gly³⁶ in δ , and Kex2p cleavage sites at the N and C termini of α and β are shown; Kex1p processing at the C terminus of β removes the terminal Arg residue and uncovers the toxin's ER retention/targeting signal (HDEL) which is essential for retrograde toxin transport in a sensitive target cell.

targeting signal to the HDEL-receptor of the target cell (Fig. 6). It will need further experiments to actually demonstrate that disulfide bond formation controls the accessibility of the HDEL signal, and by surface plasmon resonance (BIAcore analysis) we are currently determining the *in vitro* binding kinetics (association and dissociation constants) between the cellular HDEL receptor Erd2p and various mutant toxins that are defective in correct disulfide bond formation.

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REFERENCES

- Bevan, A., Brenner, C. & Fuller, R. S. (1998). Quantitative assessment of enzyme specificity *in vivo*: P2 recognition by Kex2 protease defined in a genetic system. *Proc Natl Acad Sci USA* **95**, 10384–10389.
- Boone, C., Bussey, H., Greene, D., Thomas, D. Y. & Vernet, T. (1986). Yeast killer toxin: site-directed mutations implicate the precursor protein as the immunity component. *Cell* **46**, 105–113.
- Bostian, K. A., Rogers, D. T. & Tipper, D. J. (1983). A glycosylated protoxin in killer yeast: models for its structure and maturation. *Cell* **32**, 169–180.

Bourbonnais, Y., Ash, J., Daigle, M. & Thomas, D. Y. (1993). Isolation and characterization of *S. cerevisiae* mutants defective in somatostatin expression: cloning and functional role of a yeast gene encoding an aspartyl protease in precursor processing at monobasic cleavage sites. *EMBO J* **12**, 285–294.

Brenner, C. & Fuller, R. S. (1992). Structural and enzymatic characterization of a purified prohormone-processing enzyme: secreted, soluble Kex2 protease. *Proc Natl Acad Sci USA* **89**, 922–926.

Bryant, N. J. & Boyd, A. (1993). Immunolocalization of Kex2p-containing organelles from yeast demonstrates colocalization of three processing proteinases to a single Golgi compartment. *J Cell Sci* **106**, 815–822.

Bussey, H., Sacks, W., Galley, D. & Saville, D. (1982). Yeast killer plasmid mutations affecting toxin secretion and activity and toxin immunity function. *Mol Cell Biol* **2**, 346–354.

Chaudhary, V. K., Jinno, Y., FitzGerald, D. & Pastan, I. (1990). *Pseudomonas* exotoxin contains a specific sequence at the carboxyl terminus that is required for cytotoxicity. *Proc Natl Acad Sci USA* **87**, 308–312.

Dignard, D., Whiteway, M., Germain, D., Tessier, D. & Thomas, D. Y. (1991). Expression in yeast of a cDNA copy of the K2 killer toxin gene. *Mol Gen Genet* **227**, 127–136.

Dmochowska, A., Dignard, D., Henning, D., Thomas, D. Y. & Bussey, H. (1987). Yeast KEX1 gene encodes a putative protease with a carboxypeptidase B-like function involved in killer toxin and alpha-factor precursor processing. *Cell* **50**, 573–584.

Egel-Mitani, M., Flygnering, H. P. & Hansen, M. T. (1990). A novel aspartyl protease allowing KEX2-independent MF alpha pheromone processing in yeast. *Yeast* **6**, 127–137.

Eisfeld, K., Riffer, F., Mentges, J. & Schmitt, M. J. (2000). Endocytotic uptake and retrograde transport of a virally encoded killer toxin in yeast. *Mol Microbiol* **37**, 926–940.

Fuller, R. S., Brake, A. J. & Thorner, J. (1989). Intracellular targeting and structural conservation of a prohormone-processing endoprotease. *Science* **246**, 482–486.

Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K. & Pease, L. R. (1989). Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* **77**, 61–68.

Ledgerwood, E. C., Brennan, S. O., Cawley, N. X., Loh, Y. P. & George, P. M. (1996). Yeast aspartic protease 3 (Yap3) prefers substrates with basic residues in the P2, P1 and P2' positions. *FEBS Lett* **383**, 67–71.

Martinac, B., Zhu, H., Kubalsky, A., Zhou, X.-L., Culbertson, M., Bussey, H. & Kung, C. (1990). Yeast K1 killer toxin forms ion channels in sensitive yeast spheroplasts and in artificial liposomes. *Proc Natl Acad Sci USA* **87**, 6228–6232.

Park, C. M., Bruenn, J. A., Ganesa, C., Flurkey, W. F., Bozarth, R. F. & Koltin, Y. (1994). Structure and heterologous expression of the *Ustilago maydis* viral toxin KP4. *Mol Microbiol* **11**, 155–164.

Pelham, H. R. B., Roberts, L. M. & Lord, J. M. (1992). Toxin entry: how reversible is the secretory pathway? *Trends Cell Biol* **2**, 183–185.

Redding, K., Holcomb, C. & Fuller, R. S. (1991). Immunolocalization of Kex2 protease identifies a putative late Golgi compartment in the yeast *Saccharomyces cerevisiae*. *J Cell Biol* **113**, 527–538.

Schiestl, R. H. & Gietz, R. D. (1989). High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. *Curr Genet* **16**, 339–346.

Schmitt, M. J. (1995). Cloning and expression of a cDNA copy of the viral K28 killer toxin gene in yeast. *Mol Gen Genet* **246**, 236–246.

- Schmitt, M. J. & Eisfeld, K. (1999).** Killer viruses in *S. cerevisiae* and their general importance in understanding eucaryotic cell biology. *Recent Res Devel Virol* **1**, 525–545.
- Schmitt, M. J. & Tipper, D. J. (1990).** K28, a unique double-stranded RNA killer virus of *Saccharomyces cerevisiae*. *Mol Cell Biol* **10**, 4807–4815.
- Schmitt, M. J. & Tipper, D. J. (1992).** Genetic analysis of maintenance and expression of L and M double-stranded RNAs from yeast killer virus K28. *Yeast* **8**, 373–384.
- Schmitt, M. J. & Tipper, D. J. (1995).** Sequence of the M28 dsRNA: preprotoxin is processed to an alpha/beta heterodimeric protein toxin. *Virology* **213**, 341–351.
- Schmitt, M., Brendel, M., Schwarz, R. & Radler, F. (1989).** Inhibition of DNA synthesis in *Saccharomyces cerevisiae* by yeast killer toxin KT28. *J Gen Microbiol* **135**, 1529–1535.
- Schmitt, M. J., Klavehn, P., Wang, J., Schönig, I. & Tipper, D. J. (1996).** Cell cycle studies on the mode of action of yeast K28 killer toxin. *Microbiology* **142**, 2655–2662.
- Steiner, D. F., Smeekens, S. P., Ohagi, S. & Chan, S. J. (1992).** The new enzymology of precursor processing endoproteases. *J Biol Chem* **267**, 23435–23438.
- Sturley, S. L., Elliot, Q., Le Vitre, J., Tipper, D. J. & Bostian, K. A. (1986).** Mapping of functional domains within the *Saccharomyces cerevisiae* type 1 killer preprotoxin. *EMBO J* **5**, 3381–3389.
- Tao, J., Ginsberg, I., Banerjee, N., Held, W., Koltin, Y. & Bruenn, J. A. (1990).** *Ustilago maydis* KP6 killer toxin: structure, expression in *Saccharomyces cerevisiae*, and relationship to other cellular toxins. *Mol Cell Biol* **10**, 1373–1381.
- Tipper, D. J. & Schmitt, M. J. (1991).** Yeast dsRNA viruses: replication and killer phenotypes. *Mol Microbiol* **5**, 2331–2338.
- von Heijne, G. (1986).** A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res* **14**, 4683–4690.
- Wickner, R. B. (1992).** Double-stranded and single-stranded RNA viruses of *Saccharomyces cerevisiae*. *Annu Rev Microbiol* **46**, 347–375.
- Yoshida, T., Chen, C., Zhang, M. & Wu, H. C. (1991).** Disruption of the Golgi apparatus by brefeldin A inhibits the cytotoxicity of ricin, modeccin, and *Pseudomonas* toxin. *Exp Cell Res* **192**, 389–395.
- Zhu, H., Bussey, H., Thomas, D. Y., Gagnon, J. & Bell, A. W. (1987).** Determination of the carboxyl termini of the alpha and beta subunits of yeast K1 killer toxin: requirement of a carboxypeptidase B-like activity for maturation. *J Biol Chem* **262**, 10728–10732.
- Zhu, Y. S., Zhang, X. Y., Cartwright, C. B. & Tipper, D. J. (1992).** Kex2-dependent processing of yeast K1 killer preprotoxin includes cleavage at ProArg-44. *Mol Microbiol* **6**, 511–520.

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